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Affinity Modulation of Platelet Integrin $\alpha_{IIb}\beta_3$ by β_3-Endonexin, a Selective Binding Partner of the β_3 Integrin Cytoplasmic Tail

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Abstract. Platelet agonists increase the affinity state of integrin $\alpha_{IIb}\beta_3$, a prerequisite for fibrinogen binding and platelet aggregation. This process may be triggered by a regulatory molecule(s) that binds to the integrin cytoplasmic tails, causing a structural change in the receptor. β_3-Endonexin is a novel 111–amino acid protein that binds selectively to the β_3 tail. Since β_3-endonexin is present in platelets, we asked whether it can affect $\alpha_{IIb}\beta_3$ function. When β_3-endonexin was fused to green fluorescent protein (GFP) and transfected into CHO cells, it was found in both the cytoplasm and the nucleus and could be detected on Western blots of cell lysates. PAC1, a fibrinogen-mimetic mAb, was used to monitor $\alpha_{IIb}\beta_3$ affinity state in transfected cells by flow cytometry. Cells transfected with GFP and $\alpha_{IIb}\beta_3$ bound little or no PAC1. However, those transfected with GFP/β_3-endonexin and $\alpha_{IIb}\beta_3$ bound PAC1 specifically in an energy-dependent fashion, and they underwent fibrinogen-dependent aggregation. GFP/β_3-endonexin did not affect levels of surface expression of $\alpha_{IIb}\beta_3$ nor did it modulate the affinity of an $\alpha_{IIb}\beta_3$ mutant that is defective in binding to β_3-endonexin. Affinity modulation of $\alpha_{IIb}\beta_3$ by GFP/β_3-endonexin was inhibited by coexpression of either a monomeric β_3 cytoplasmic tail chimera or an activated form of H-Ras. These results demonstrate that β_3-endonexin can modulate the affinity state of $\alpha_{IIb}\beta_3$ in a manner that is structurally specific and subject to metabolic regulation. By analogy, the adhesive function of platelets may be regulated by such protein–protein interactions at the level of the cytoplasmic tails of $\alpha_{IIb}\beta_3$.

INTEGRINS are $\alpha\beta$ heterodimers and each subunit contains a relatively large extracellular domain, a membrane-spanning domain, and a 20–70–amino acid cytoplasmic tail. They function in cell adhesion and signaling by interacting with extracellular matrix proteins or cellular counter-receptors on the one hand, and with intracellular proteins on the other (8, 34, 59). The adhesive function of many integrins is subject to rapid regulation by two processes collectively referred to as "inside-out" signaling: (*a*) a structural change intrinsic to the heterodimer, and (*b*) clustering of heterodimers within the plane of the plasma membrane. The former modulates the affinity of the ligand–receptor interaction and thus is often referred to as "affinity modulation." The latter increases the valency and, therefore, the avidity of the interaction. These two types of regulation are not mutually exclusive, and their relative contributions probably vary with the integrin and the cell type (12, 20, 62, 71).

A good example of the pathophysiological significance of rapid integrin regulation involves platelet $\alpha_{IIb}\beta_3$. Circulating platelets ordinarily do not interact with each other or with the blood vessel wall. However, when the vessel is damaged by trauma or disease, platelets become activated and $\alpha_{IIb}\beta_3$ is converted within seconds into a functional receptor for several Arg-Gly-Asp–containing ligands, including fibrinogen and von Willebrand factor. Since ligand binding is required for platelet aggregation, inside-out signaling is a prerequisite for primary hemostasis and for formation of occlusive platelet thrombi in vascular diseases (9, 27). Affinity modulation is thought to be responsible for the initial, reversible phase of fibrinogen binding to platelets, while integrin clustering may be involved in stabilizing the interaction (14, 52).

Studies with intact and permeabilized platelets indicate that specific intracellular mediators promote rapid increases or decreases in ligand binding to $\alpha_{IIb}\beta_3$. Excitatory platelet agonists, such as thrombin, increase ligand binding by a process that involves heterotrimeric G proteins and protein and lipid kinases (38, 61, 69, 74). On the other hand, substances such as prostacyclin and nitric oxide,

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which stimulate protein kinase A and protein kinase G, respectively, inhibit or reverse ligand binding (22, 28). In addition to intracellular mediators, the cytoplasmic tails of $\alpha_{IIb}\beta_3$ appear to participate in the regulation of fibrinogen binding. Platelets from patients with variant forms of Glanzmann thrombasthenia due to a deletion or mutation in the β_3 cytoplasmic tail fail to aggregate in response to agonists despite near normal levels of $\alpha_{IIb}\beta_3$ (6; Wang, R., D.R. Ambruso, and P.J. Newman. 1994. *Blood.* 84:244*a*). However, it is not clear how intracellular signals affect the cytoplasmic tails of $\alpha_{IIb}\beta_3$ or how changes at the level of these tails regulate ligand binding. One hypothesis is that specific intracellular proteins bind to the tails and promote a structural change that is propagated across the plasma membrane to the extracellular face of the receptor. Accordingly, recent efforts have focused on identifying proteins that interact with integrin cytoplasmic tails (11).

Using a yeast two-hybrid screening strategy, we recently discovered a novel 111–amino acid polypeptide called β_3-endonexin, which is capable of binding to the cytoplasmic tail of the β_3 integrin subunit, both in yeast and in vitro (63). However, it fails to bind to other integrin tails, including those of β_1, β_2, and α_{IIb}. Since β_3-endonexin is expressed in platelets, the present studies were carried out to determine whether this protein can modulate the ligand-binding function of $\alpha_{IIb}\beta_3$. Using a CHO cell model system to transiently express $\alpha_{IIb}\beta_3$ and β_3-endonexin, we now report that this protein can increase the affinity state and the adhesive function of $\alpha_{IIb}\beta_3$. Moreover, these effects are structurally specific and subject to metabolic regulation.

Materials and Methods

Reagents

Mammalian expression vectors for green fluorescent protein (GFP)[1] (pS65T-C1 and pEGFP-C1) were obtained from Clontech (Palo Alto, CA). Monoclonal antibodies PAC1, A2A9, D57, anti-LIBS1, and anti-LIBS6 were obtained from ascites and purified as described (30). PAC1 was conjugated to phycoerythrin (PE) by first derivatizing it with *N*-succinimidyl *S*-acetylthioacetate (Pierce Chemical Co., Rockford, IL). SH groups were deprotected with hydroxylamine, and the antibody was then coupled to PE that had been derivatized with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co.). PE:PAC1 conjugates (1:1 mol/mol) were isolated by sizing on a Superose 6 column (Pharmacia Fine Chemicals, Piscataway, NJ). In one experiment, PAC1 IgMκ was first reduced to the 185-kD monomer at pH 8.6 with 20 mM cysteine before conjugating to PE (46).

DNA Constructs

To express β_3-endonexin as a protein fused to the carboxy terminus of GFP, β_3-endonexin cDNA was excised from a yeast expression vector with XbaI and BamHI (63), and the recessed 3′ termini were filled in using Klenow (Boehringer Mannheim Biochemicals, Indianapolis, IN). The GFP vectors, pS65T-C1 and pEGFP-C1, were cut with XhoI, blunt-ended with Klenow, and ligated to β_3-endonexin with T4 DNA ligase (Boehringer Mannheim Biochemicals). After transformation of DH5α, clones in the correct orientation were selected by PCR using a sense primer in GFP and an antisense primer in β_3-endonexin.

Plasmid DNA encoding the cytoskeletal protein, VASP, was a gift from Ulrich Walter and Thomas Jarchau (25) (Medizinische Universitatsklinik, Wurzburg, Germany). The coding sequence of VASP was amplified with Pfu polymerase (Stratagene, La Jolla, CA) using a sense primer containing an XhoI site and an antisense primer with an HindIII site. The digested PCR fragment was subcloned into XhoI- and HindIII-cut pEGFP-C1 so that VASP would be expressed in-frame at the carboxy terminus of GFP. Plasmid DNA encoding FRNK, an autonomously expressed carboxy-terminal segment of $pp125^{FAK}$, was a gift from Michael Schaller (University of North Carolina, Chapel Hill, NC) (57). FRNK was amplified with Pfu polymerase with a sense primer containing a BglII site and an antisense primer containing an EcoRI site. The digested PCR fragment was subcloned into BglII- and EcoRI-cut pEGFP-C1 so that FRNK would be expressed in-frame at the carboxy terminus of GFP.

pCDM8 expression vectors encoding wild-type αIIb, β_3, a mutant form of β_3 containing a single amino acid substitution (S752P), and H-Ras (G12V) have been described (33, 49). Tac-β_3 and Tac-α_5 chimeras were in the vector, CMV-IL2R (7). All expression plasmids were amplified in *Escherichia coli* and purified (Plasmid Maxi Kit; Qiagen, Inc., Chatsworth, CA). Before use in transfection experiments, each plasmid was sequenced in the Scripps Research Institute DNA Core Facility to confirm the authenticity of the coding sequences.

Transient Protein Expression in CHO Cells

cDNAs were transfected into CHO-K1 cells with Lipofectamine (GIBCO BRL, Gaithersburg, MD). A total of 5 μg of plasmid DNA and 20 μl of Lipofectamine solution was incubated for 10 min in 200 μl of DME and then diluted with 3.8 ml of DME. Unless otherwise indicated, the amount of DNA per transfection included 0.5 μg each of αIIb and β_3 and varying amounts of the GFP plasmids to obtain equivalent degrees of GFP expression (e.g., 4 μg of pS65T, 4 μg of pS65T/β_3-endonexin, 0.02 μg of pEGFP, 0.2 μg of pEGFP/β_3-endonexin, or 0.05 μg of pEGFP/VASP). When necessary, an empty vector (pcDNA3; Invitrogen, San Diego, CA) was included to equalize the amount of DNA transfected. In some experiments, 2 μg of the Tac-β_3, Tac-α5, or H-Ras (G12V) plasmid was cotransfected along with pEGFP/β_3-endonexin and the plasmids for αIIb and β_3. DNA/Lipofectamine mixtures were added to CHO cells at 30–50% confluence in a 100-mm tissue-culture plate. 6 h later, the medium was changed to DME containing 10% FBS, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. 48 h after transfection, the cells were evaluated biochemically and by flow cytometry.

Evaluation of GFP/β_3-Endonexin Expression in CHO Cells

Expression of GFP/β_3-endonexin fusion proteins was confirmed by immunoblotting. 48 h after transfection, the cells were lysed for 30 min at 4°C in a lysis buffer containing 1% Triton X-100, 0.9% NaCl, 1 mM $CaCl_2$, 50 mM Tris, pH 7.2, and protease inhibitors (100 U/ml aprotinin, 0.5 mM leupeptin, 4 mM Pefabloc) (63). After clarification of the lysate in a microfuge, protein concentration was determined with a bicinchoninic acid reagent (BCA; Pierce Chemical Co.). 30 μg of each sample was then electrophoresed in 14% SDS-polyacrylamide gels under reducing conditions. After transfer to nitrocellulose, immunoblotting was performed with a rabbit polyclonal antibody reactive with GFP (Clontech) or rabbit antibodies reactive with β_3-endonexin (63). After the addition of affinity-purified, HRP-conjugated goat anti–rabbit IgG, the blots were developed for 0.1–1 min using the enhanced chemiluminescence reaction (ECL; Amersham Corp., Arlington Heights, IL).

To study the binding of GFP/β_3-endonexin to the β_3 integrin cytoplasmic tail, the 47–amino acid β_3 tail was expressed in bacteria with a $(His)_6$ tag at its amino terminus (pET His Tag System; Novagen, Inc., Madison, WI), and then immobilized on a nickel-agarose matrix. Transiently transfected CHO cells expressing equivalent amounts of GFP/β_3-endonexin or GFP were lysed in 0.4 ml of the Triton X-100 lysis buffer. After clarification, 0.35 ml was diluted with an equal volume of lysis buffer containing no Triton, and each sample was batch-incubated with 0.1 ml packed volume of the His-β_3 tail affinity matrix for 12 h at 4°C while shaking. After washing the matrices five times with 10 vol of lysis buffer, bound proteins were eluted into 0.7 ml of lysis buffer by addition of 1 M imidazole. Samples were electrophoresed on 12% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose. Immunoblotting was performed with the polyclonal anti-GFP antibody.

To evaluate whether GFP/β_3-endonexin could be specifically coimmunoprecipitated with the β_3 integrin subunit from CHO cells, immunoprecipitation studies were carried out in the Trition X-100 lysis buffer essentially as described (30). The β_3 integrin subunit was immunoprecipitated from 2-mg aliquots of cell lysate using 10 μg/ml of the murine mAb, SSA6,

1. *Abbreviations used in this paper*: GFP, green fluorescent protein; PE, phycoerythrin.

and protein A–Sepharose (1). Control immunoprecipitations were carried out with affinity-purified mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) and with an isotype-matched mAb against von Willebrand factor, RG 7 (a gift from Zaverio Ruggeri; Scripps Research Institute, La Jolla, CA). Samples were electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions and subsequent Western blots were probed with the polyclonal anti-GFP antibody. To demonstrate equivalent recovery of the β_3 integrin subunit in the immunoprecipitates, blots were stripped and reprobed with Ab 8035, a rabbit polyclonal antibody specific for β_3.

Evaluation of $\alpha_{IIb}\beta_3$ Affinity State

48 h after transfection, CHO cells were resuspended at 1–2 $\times$ 10^6 cells per ml in Tyrode's buffer containing 2 mM $CaCl_2$ and $MgCl_2$ (49). Cells were then incubated in the dark in 50-μl aliquots with 20 μg/ml PE-PAC1 for 30 min at room temperature. Some samples also contained an anti-β_3 antibody (2% anti-LIBS6 ascites) that stabilizes $\alpha_{IIb}\beta_3$ in a high affinity state (30). Others contained an $\alpha_{IIb}\beta_3$-selective inhibitor of ligand binding (either 2 μM Ro 43-5054 or 10 μM Integrilin) (2, 56). Samples were then diluted with 0.5 ml Tyrode's buffer containing 10 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and analyzed on a FACS®can or FACS®Calibur flow cytometer (Becton Dickinson, San Jose, CA). In one set of experiments, the cells were preincubated for 30 min at room temperature with 4 mg/ml of 2-deoxy-D-glucose (Sigma Chemical Co.) and 0.2% sodium azide before incubation with PE-PAC1.

After electronic compensation of the FL1, FL2, and FL3 fluorescence channels, PE-PAC1 binding (FL2) was analyzed on the gated subset of live cells (propidium iodide–negative, FL3 channel) that were positive for GFP expression (FL1 channel). PAC1 binding was expressed as an "activation index" calculated from median fluorescence intensity measurements (49). The activation index is defined as 100 $\times$ (Fx-Fi)/(Fm-Fi), where Fx is PAC1 fluorescence in the absence and Fi is PAC1 fluorescence in the presence of Ro 43-5054 or Integrilin. Fm is PAC1 fluorescence in the presence of anti-LIBS6.

Fibrinogen Binding Assay

Fibrinogen binding to GFP-positive cells was determined by flow cytometry using biotinylated anti-LIBS1, which recognizes a fibrinogen-sensitive epitope on the β_3 subunit (19). Cells were prepared as for the PAC1 binding studies and incubated for 30 min in the dark at room temperature with fibrinogen (250 μg/ml; Enzyme Research Laboratories, South Bend, IN), biotin-LIBS1 (20 μg/ml), and phycoerythrin-streptavidin (4% final dilution; Molecular Probes, Inc., Eugene, OR). To calculate the activation index, some aliquots were incubated with anti-LIBS6 to induce maximal fibrinogen binding, while others were incubated with the function-blocking anti-$\alpha_{IIb}\beta_3$ αntibody, A2A9, to determine nonspecific fibrinogen binding. Cells were then diluted with Tyrode's buffer containing 10 μg/ml propidium iodide, and analyzed by flow cytometry.

CHO Cell Aggregation Assay

Fibrinogen-dependent aggregation of CHO cells was quantitated by flow cytometry as described (16), with minor modifications. First, CHO cells stably expressing $\alpha_{IIb}\beta_3$ (49) were labeled with a red fluorescent tracer, hydroxyethidine (Polysciences Inc., Junction City, OR). Then 250 μl of these cells (4×10^6/ml) were added to siliconized glass cuvettes containing 250 μl of cells (2×10^6/ml) that had been transfected with GFP/β_3-endonexin (or GFP) and $\alpha_{IIb}\beta_3$. After addition of 300 μg/ml fibrinogen, the cells were stirred with a magnetic stir bar at 1,000 rpm for 20 min at room temperature. In some cases, the incubations with fibrinogen were also carried out in the presence of 20 μg/ml A2A9 or 10 μM Integrilin to inhibit fibrinogen binding. Incubations were stopped by addition of 0.25% formaldehyde, and the samples were kept on ice for 30 min before flow cytometric detection of mixed red–green cellular aggregates.

Subcellular Localization of GFP/β_3-Endonexin

48 h after transfection, CHO cells were cultured on fibrinogen-coated coverslips for 2 h at 37°C, and then processed and analyzed by fluorescence microscopy for expression of GFP, α_{IIb}, and β_3 as described (32). HMEC-1 human endothelial cells, which express $\alpha_V\beta_3$, were similarly cultured on fibrinogen-coated coverslips, and then microinjected with plasmid DNA (0.5 μg/μl) encoding various GFP proteins (45). After 4 h at 37°C, the cells were processed and analyzed by fluorescence microscopy for expression of GFP, α_V, and β_3.

Results

Expression of β_3-Endonexin in CHO Cells

CHO cells provide a useful model system for characterizing the adhesive and signaling functions of ectopically expressed $\alpha_{IIb}\beta_3$ (43, 49, 50). Therefore, β_3-endonexin was transiently coexpressed with $\alpha_{IIb}\beta_3$ in these cells to study its effects on the ligand-binding function of this integrin. β_3-Endonexin cDNA was fused in-frame to the 3′ end of two different versions of GFP in a mammalian expression plasmid. One form (S65T) is red-shifted and the other (EGFP) is both red-shifted and codon-optimized for mammalian expression. 48 h after transfection, expression of recombinant proteins was assessed by Western blotting of cell lysates. GFP/β_3-endonexin was detectable using an anti-GFP antibody, and the codon-optimized plasmid provided higher levels of protein expression for a given amount of DNA transfected (Fig. 1). Subsequently, therefore, the amount of each plasmid used was adjusted to obtain roughly equivalent amounts of GFP/β_3-endonexin expression, and the plasmids were used interchangeably in the following experiments. GFP/β_3-endonexin was also detectable with polyclonal antibodies raised against either recombinant human β_3-endonexin or a synthetic peptide consisting of the carboxy-terminal 17 residues of the protein (Fig. 1). No hamster protein cross-reactive with these antibodies was detected in CHO cells. These results indicate that full-length GFP/β_3-endonexin can be expressed in CHO cells.

Previous studies have shown that β_3-endonexin binds in vitro to the β_3 integrin subunit from detergent-solubilized platelets and CHO cells (13, 63). To determine if β_3-endonexin retains its ability to bind to the β_3 integrin subunit after its fusion to GFP, lysates from CHO cells expressing GFP/β_3-endonexin were passed over an affinity matrix containing the bacterially expressed β_3 cytoplasmic tail. GFP/β_3-endonexin, but not GFP, was specifically retained by and eluted from this affinity matrix (Fig. 2 *A*). Moreover, GFP/β_3-endonexin and the β_3 integrin subunit could be specifically coprecipitated from CHO cell lysates (Fig. 2 *B*). Finally, CHO cells containing GFP/β_3-endonexin were strongly fluorescent in the FL1 channel of a flow cytometer (see below). Thus, fusion of β_3-endonexin to the carboxy terminus of GFP abrogates neither the integrin-binding function of β_3-endonexin nor the fluorescent properties of GFP.

β_3-Endonexin Increases the Affinity State of Integrin $\alpha_{IIb}\beta_3$

48 h after cotransfection of CHO cells with expression plasmids encoding $\alpha_{IIb}\beta_3$ αnd GFP/β_3-endonexin, the affinity state of $\alpha_{IIb}\beta_3$ was determined by flow cytometry using a PE conjugate of the fibrinogen-mimetic mAb, PAC1. Since transfection efficiencies varied from 15–45%, data acquisition included only live cells positive for GFP fluorescence. About 75% of these cells were also positive for $\alpha_{IIb}\beta_3$, as assessed by staining with an antibody specific for the $\alpha_{IIb}\beta_3$ complex (D57). To standardize the results of PAC1 binding from experiment to experiment, binding

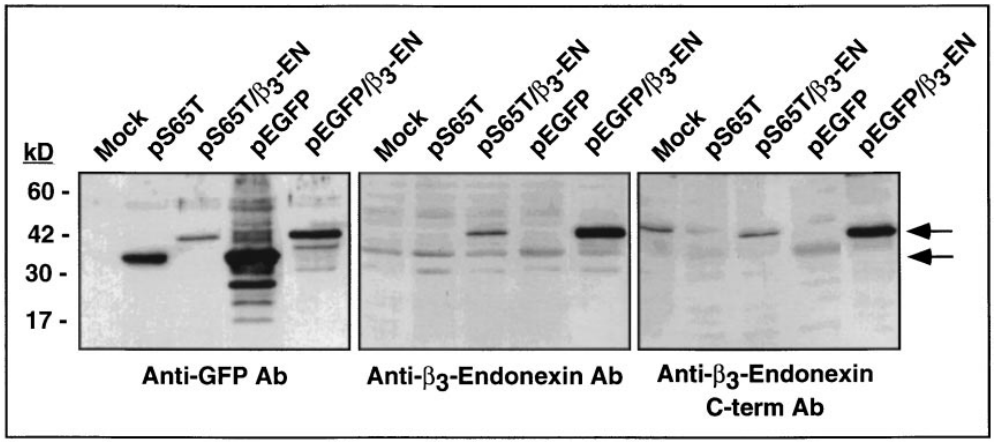

Figure 1. Expression of GFP and GFP/β_3-endonexin in CHO cells. CHO cells were either mock transfected or transfected with 4 μg of the indicated plasmids as described in Materials and Methods. 48 h later, the cells were lysed in a buffer containing Triton X-100, and 30 μg of each sample was probed on Western blots with the indicated polyclonal antibodies. (*Upper arrow*) Position of GFP/β_3-endonexin; (*lower arrow*) position of GFP. The band in the "mock" lane stained with anti–β_3-endonexin carboxy-terminal antibody migrated more slowly than GFP/β_3-endonexin and was nonspecific.

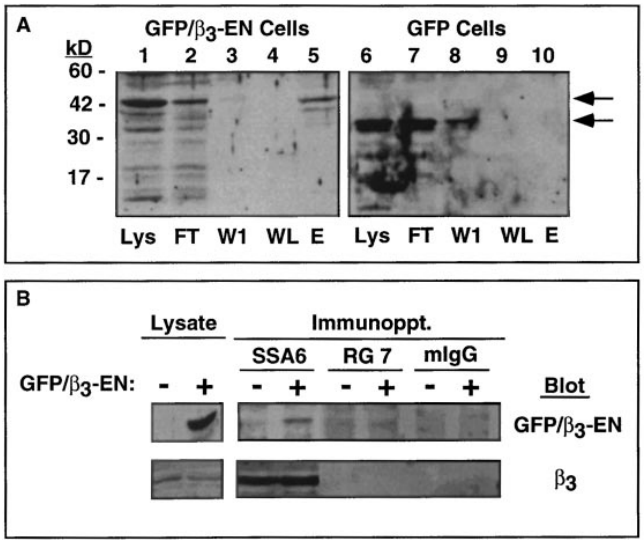

Figure 2. Interactions of GFP/β_3-endonexin with the β_3 integrin cytoplasmic tail. (*A*) CHO cells were transfected with 4 μg of pS65T-GFP/β_3-endonexin or pS65T-GFP. 48 h later, the cells were lysed in a Triton X-100–containing buffer, and equal volumes of the lysates were batch-incubated with a His-β_3 cytoplasmic tail affinity matrix. After extensive washing, proteins were eluted with 1 M imidazole, and equal volumes of the indicated fractions were transferred to nitrocellulose and probed with a polyclonal antibody to GFP. (*Upper arrow*) Position of GFP/β_3-endonexin; (*lower arrow*) position of GFP. Previous studies of this kind have already documented that β_3-endonexin interacts with the β_3 but not the β_1 cytoplasmic tail (63). (*B*) CHO cells expressing both $\alpha_{IIb}\beta_3$ and GFP/β_3-endonexin (+ *GFP/β_3-EN*) or $\alpha_{IIb}\beta_3$ alone (− *GFP/β_3-EN*) were lysed in Triton X-100 lysis buffer, and clarified lysates were immunoprecipitated either with a monoclonal anti-β_3 antibody (*SSA6*), an isotype control antibody (*RG 7*), or mouse IgG (*mIgG*). Western blotting was performed initially with a polyclonal antibody to GFP and blots were then reprobed with a polyclonal anti-β_3 antibody. The first two lanes represent 30 μg of lysate. *Lys*, lysate; *FT*, flow-through; *W1*, first wash; *WL*, last wash; *E*, eluate.

was expressed as an activation index calculated from median fluorescence values (49). To obtain this index, nonspecific PAC1 binding was determined in the presence of a selective inhibitor of ligand binding to $\alpha_{IIb}\beta_3$ (either Ro 43-5054 or Integrilin). Maximal PAC1 binding was determined in the presence of an activating anti-β_3 antibody (anti-LIBS6) (49). After subtraction of nonspecific binding, this maximal binding was assigned an activation index of 100. Consequently, the activation index for PAC1 binding can range from 0 to 100.

Fig. 3 shows the results of a representative experiment. PAC1 binding to CHO cells transfected with GFP/β_3-endonexin and $\alpha_{IIb}\beta_3$ exhibited a relatively high activation index of 44 (Fig. 3 *A*). In contrast, PAC1 binding to cells transfected with GFP and $\alpha_{IIb}\beta_3$ exhibited a lower activation index of 18 (Fig. 3 *D*), a value similar to that observed previously for $\alpha_{IIb}\beta_3$ transfectants in the absence of GFP (31, 49). Thus, expression of β_3-endonexin appears to activate $\alpha_{IIb}\beta_3$ and increase its affinity for a cognate ligand.

This impression was confirmed by the series of experiments summarized in Fig. 4. Compared with cells expressing GFP, those expressing GFP/β_3-endonexin consistently showed an increase in PAC1 binding, and the difference was statistically significant ($P < 0.03$). In contrast, PAC1 binding to cells expressing an unrelated GFP fusion protein, GFP/VASP, was not increased despite similar levels of recombinant protein expression. VASP was chosen because it is present in platelets and localizes to integrin-rich focal adhesions (25). Although not shown, the PAC1 activation index for GFP/β_3-endonexin cells (44 ± 5) began to approach that for cells expressing a constitutively active form of $\alpha_{IIb}\beta_3$ ($\alpha_{IIb}\alpha_{6A}\beta_3$; 61 ± 6; $n = 3$) (49). Expression of GFP/β_3-endonexin or the other GFP proteins did not affect levels of surface expression of $\alpha_{IIb}\beta_3$, as determined by the binding of antibody D57. All together, these results indicate that expression of β_3-endonexin can increase the affinity state of $\alpha_{IIb}\beta_3$.

Platelets containing $\alpha_{IIb}\beta_3$ with a specific point mutation in the β_3 cytoplasmic tail at position 752 (S→P) fail to bind

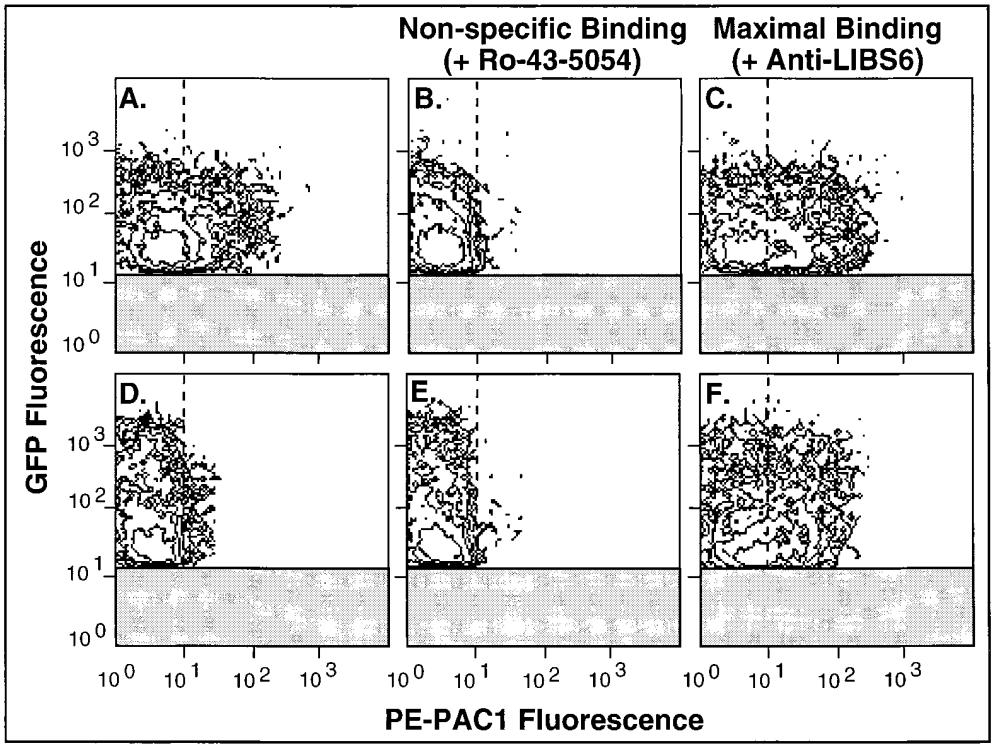

Figure 3. Effect of GFP/β_3-endonexin on PAC1 binding to $\alpha_{IIb}\beta_3$ in CHO cells. CHO cells were transfected with $\alpha_{IIb}\beta_3$ and either GFP/β_3-endonexin (contour plots *A*, *B*, and *C*) or GFP (plots *D*, *E*, and *F*). 48 h later, binding of PE-PAC1 (x-axis) to green fluorescent–positive cells (y-axis) was analyzed by flow cytometry. Each contour plot represents 10,000 cells. Plots *B* and *E* represent nonspecific PAC1 binding determined in the presence of Ro 43-5054. Plots *C* and *F* represent maximal PAC1 binding in the presence of an activating anti-β_3 antibody, anti-LIBS6. Note that there was more PAC1 binding to GFP/β_3-endonexin cells (plot *A*) than to GFP cells (plot *D*), a conclusion supported by the calculated activation indices (plot *A*, activation index = 44%; plot *D*, activation index = 18%).

fibrinogen or aggregate (6). Furthermore, the binding of β_3-endonexin to this mutant β_3 integrin subunit is markedly reduced (63). When $\alpha_{IIb}\beta_3$ (S752P) was coexpressed with GFP/β_3-endonexin in CHO cells, no increase in PAC1 binding was observed (Fig. 4). This suggests that β_3-endonexin modulates the affinity state of $\alpha_{IIb}\beta_3$ in a structurally specific manner.

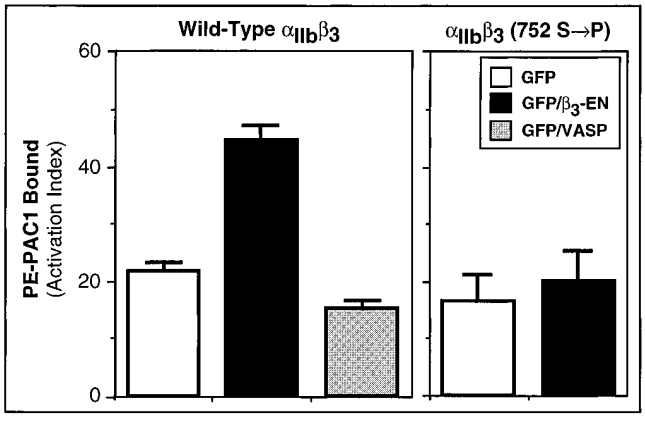

Figure 4. GFP/β_3-endonexin causes activation of $\alpha_{IIb}\beta_3$ in a structurally specific manner. PAC1 binding to transfected CHO cells was analyzed by flow cytometry as described in Materials and Methods and in the legend to Fig. 3. (*Left*) CHO cells were cotransfected with wild-type $\alpha_{IIb}\beta_3$ and either GFP (*open bar*), GFP/β_3-endonexin (*black bar*), or GFP/VASP (*shaded bar*). PAC1 binding was expressed as an activation index, and the data represent means ± SEM for 15 experiments with GFP and GFP/β_3-endonexin and three experiments with GFP/VASP. (*Right*) CHO cells were cotransfected with the signaling-deficient integrin mutant, $\alpha_{IIb}\beta_3$ (S752P), and either GFP (*open bar*) or GFP/β_3-endonexin (*black bar*). Data represent the means ± SEM of three experiments.

Functional Consequences of Integrin Affinity Modulation by β_3-Endonexin

To determine whether the changes in PAC1 binding induced by GFP/β_3-endonexin translate into increased binding of a physiological ligand, the binding of fibrinogen to CHO cells was studied by flow cytometry. Bound fibrinogen was detected with a biotinylated mAb (anti-LIBS1) specific for a fibrinogen-sensitive epitope on the β_3 subunit (19). Specific fibrinogen binding was defined as that inhibitable by a function-blocking anti-$\alpha_{IIb}\beta_3$ antibody, A2A9. CHO cells expressing wild-type $\alpha_{IIb}\beta_3$ bind little or no fibrinogen at a saturating concentration of ligand (250 μg/ml) (48). The same was true for cells expressing GFP and $\alpha_{IIb}\beta_3$. However, those expressing GFP/β_3-endonexin and $\alpha_{IIb}\beta_3$ bound increased amounts of fibrinogen (Fig. 5). Similar results were obtained when fibrinogen binding was measured directly with biotinylated fibrinogen (not shown). Thus, expression of GFP/β_3-endonexin can lead to an increase in fibrinogen binding to $\alpha_{IIb}\beta_3$.

When fibrinogen binds to activated $\alpha_{IIb}\beta_3$ on the surface of platelets or CHO cells under stirring conditions, the cells aggregate (4, 16). To determine whether GFP/β_3-endonexin can trigger this aggregation response, CHO cells expressing GFP/β_3-endonexin and $\alpha_{IIb}\beta_3$ were mixed with cells containing $\alpha_{IIb}\beta_3$ and a red fluorescent tracer, hydroxyethidine. After stirring for 20 min in the presence of 300 μg/ml fibrinogen, the formation of mixed, red–green cellular aggregates was monitored by flow cytometry. The

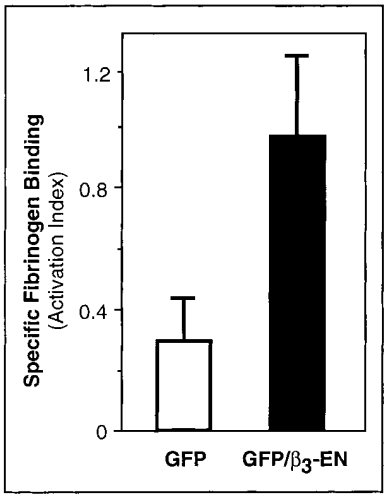

Figure 5. GFP/β_3-endonexin induces fibrinogen binding to $\alpha_{IIb}\beta_3$. 48 h after transfection of CHO cells with $\alpha_{IIb}\beta_3$ and either GFP (*open bar*) or GFP/β_3-endonexin (*black bar*), the cells were resuspended in Ca^{2+}- and Mg^{2+}-containing Tyrode's buffer and incubated for 30 min at room temperature in the presence of 250 μg/ml fibrinogen. Then fibrinogen binding to the transfected cells was assessed by flow cytometry using phycoerythrin-streptavidin and a biotinylated anti-β_3 antibody (anti-LIBS 1) sensitive to the presence of bound fibrinogen. Specific binding was defined as that blocked by antibody A2A9 (20 μg/ml), and it was expressed as an activation index. Data represent the means ± SEM of two experiments, each performed in triplicate.

rationale for this experimental design is that if fibrinogen first becomes bound to activated $\alpha_{IIb}\beta_3$ on the GFP/β_3-endonexin cells, this cell-bound fibrinogen should then be able to recruit the red fluorescent cells into mixed aggregates, even though the $\alpha_{IIb}\beta_3$ on the red fluorescent cells is initially in a low affinity state (Fig. 6 *A*) (16).

In the experiment shown in Fig. 6 *B*, it can be seen that GFP/β_3-endonexin promoted the formation of mixed aggregates (*center*), an effect that could be inhibited by the function-blocking antibody, A2A9 (*right*), or the cyclic peptide, Integrilin (not shown). In three such experiments, an average of 7.0 ± 1.6% of the cells expressing GFP/β_3-endonexin were engaged in red–green aggregates, compared with 3.5 ± 1.9% of cells expressing GFP. While this effect may seem small, it was statistically significant ($P < 0.01$). Moreover, it should be emphasized that the extent of mixed aggregation was limited by the required use of red fluorescent cells expressing low affinity $\alpha_{IIb}\beta_3$. These results indicate that affinity modulation of $\alpha_{IIb}\beta_3$ by β_3-endonexin can cause fibrinogen-dependent cell aggregation.

Factors That Influence Integrin Activation by β_3-Endonexin

Additional experiments were conducted to clarify the mechanism of action of GFP/β_3-endonexin. Although PAC1 is a multimeric IgM antibody, GFP/β_3-endonexin was also found to increase the binding of a monomeric form of PAC1 obtained by enzyme digestion. In addition, PAC1 binding because of GFP/β_3-endonexin was not affected by preincubation of the cells with 10 μM cytochalasin D, an inhibitor of actin polymerization (data not shown). Since actin polymerization promotes integrin clustering (12, 71), which would be expected to influence preferentially the binding of multivalent ligands, these results suggest that GFP/β_3-endonexin is primarily a modulator of $\alpha_{IIb}\beta_3$ affinity rather than avidity.

Next, GFP/β_3-endonexin was studied in CHO cells expressing both $\alpha_{IIb}\beta_3$ and a β_3 cytoplasmic tail chimera containing the extracellular and transmembrane domains of the Tac subunit of the IL-2 receptor. We reasoned that the chimera, which does not dimerize with α_{IIb} (7, 40), would compete intracellularly with $\alpha_{IIb}\beta_3$ for β_3-endonexin. If so, it should prevent β_3-endonexin from binding to and modulating the function of $\alpha_{IIb}\beta_3$. Indeed, expression of the Tac/β_3 chimera prevented GFP/β_3-endonexin from activating $\alpha_{IIb}\beta_3$ (Fig. 7). In contrast, a Tac chimera containing the structurally unrelated α_5 cytoplasmic tail exhibited no such effect. This is consistent with the idea that β_3-endonexin modulates integrin affinity through an interaction with the β_3 cytoplasmic tail.

Affinity modulation of $\alpha_{IIb}\beta_3$ by platelet agonists requires metabolic energy (68). In CHO cells, PAC1 binding induced by GFP/β_3-endonexin was not observed if the cells were pretreated with sodium azide and 2-deoxy-D-glucose to inhibit oxidative metabolism (Fig. 7). In this respect, the effect of β_3-endonexin in the CHO cell system is similar to that of excitatory agonists in the platelet system.

In platelets, heterotrimeric GTP-binding proteins have been implicated in affinity modulation. On the other hand, the role of the small GTPase, H-Ras, which is also present in these cells, has not been examined. Recently, Hughes and co-workers found that a constitutively active form of H-Ras (G12V) acts as a general suppressor of integrin adhesive function in CHO cells (33). Similarly, we found that the expression of H-Ras (G12V) inhibited the effects of GFP/β_3-endonexin on PAC1 binding (Fig. 7). Taken together with the energy depletion experiments, this indicates that the function of GFP/β_3-endonexin is subject to metabolic regulation.

Subcellular Localization of β_3-Endonexin

In order for β_3-endonexin to directly influence the function of the β_3 integrin cytoplasmic tail, these proteins must be located together in the cell. To address this question, HMEC-1 human endothelial cells, which attach and spread on immobilized fibrinogen through $\alpha_V\beta_3$, were microinjected with DNA encoding GFP/β_3-endonexin or GFP. 4 h later, specific green fluorescence could be observed diffusely in the cytoplasm and the nucleus. The degree of nuclear fluorescence was much greater in the case of GFP/β_3-endonexin (Fig. 8). An identical pattern of GFP/β_3-endonexin localization was observed in CHO cells that had been allowed to spread on fibrinogen through $\alpha_{IIb}\beta_3$ (not shown). These results are consistent with a generalized cytoplasmic distribution of GFP/β_3-endonexin and with a nuclear localization that may be promoted by a consensus nuclear localization signal in β_3-endonexin (see Discussion).

When CHO cells containing $\alpha_V\beta_3$ or $\alpha_{IIb}\beta_3$ are allowed to spread on fibrinogen, the β_3 cytoplasmic tail is neces-

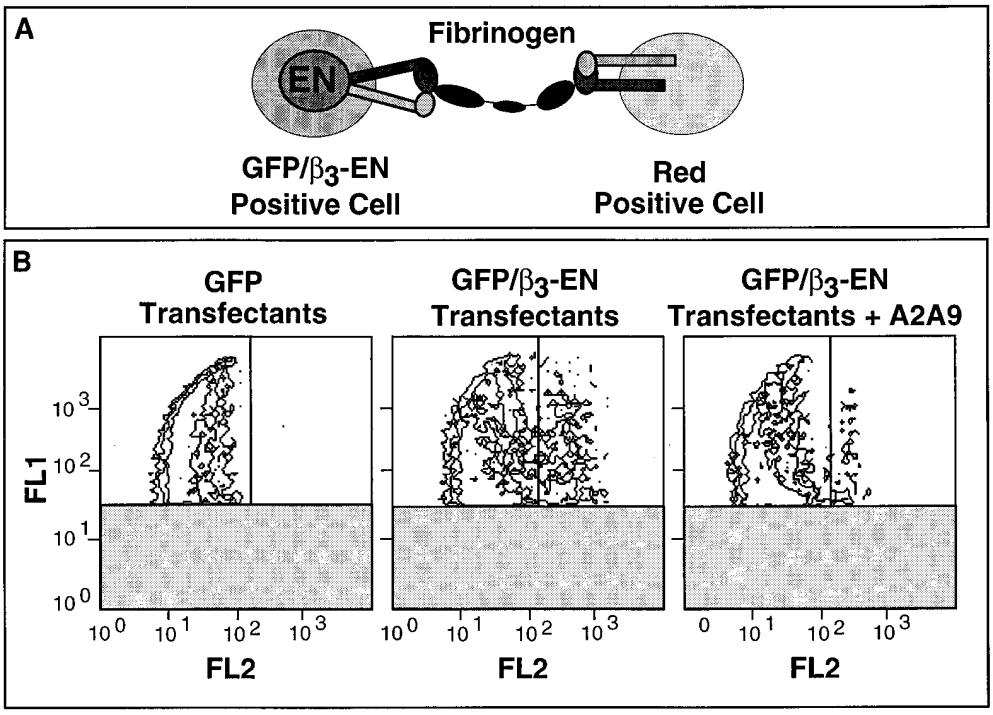

Figure 6. GFP/β_3-endonexin causes fibrinogen-dependent aggregation of CHO cells. *A* illustrates the rationale for this aggregation protocol, which is discussed in the text. In *B*, as detailed in Materials and Methods, CHO cells that had been transfected with $\alpha_{IIb}\beta_3$ and GFP (*left*) or with $\alpha_{IIb}\beta_3$ and GFP/β_3-endonexin (*center* and *right*) were mixed with CHO cells that had been stably transfected with $\alpha_{IIb}\beta_3$, and then stained with the red fluorescent dye, hydroxyethidine. After stirring for 20 min in the presence of 300 μg/ml fibrinogen, the cells were fixed with formaldehyde, and 10,000 propidium iodide–negative and GFP-positive cells (y-axis) were analyzed by flow cytometry. (*B*, *right*) The incubation with fibrinogen was carried out in the presence of 20 μg/ml antibody A2A9 to inhibit fibrinogen binding. Mixed red–green cellular aggregates appear to the right of the vertical line on the FL2 axis.

sary and sufficient for localization of the β_3 integrins to focal adhesions (40, 72). Immunostaining of HMEC-1 cells revealed that α_V and β_3 were localized both in a diffuse pattern consistent with a generalized plasma membrane distribution and in discrete foci characteristic of focal adhesions (Fig. 9). There was no strong or consistent localization of GFP/β_3-endonexin to these focal adhesions, excluding the possibility that β_3-endonexin might associate tightly with the β_3 cytoplasmic tail during cytoskeletal assembly. However, some weak staining of β_3-endonexin in focal adhesions was observed, suggesting that a weaker or more transient association may occur (Fig. 9, *arrowheads*). No localization of GFP to focal adhesions was detected. As a positive control, GFP was fused to FRNK, an autonomously expressed segment of pp125^{FAK} that contains a focal adhesion targeting sequence (57). After microinjection, GFP/FRNK significantly localized to focal adhesions, demonstrating that a GFP fusion protein can target to these structures under the experimental conditions used here (Fig. 8). Thus, GFP/β_3-endonexin is not strongly or consistently concentrated in focal adhesions.

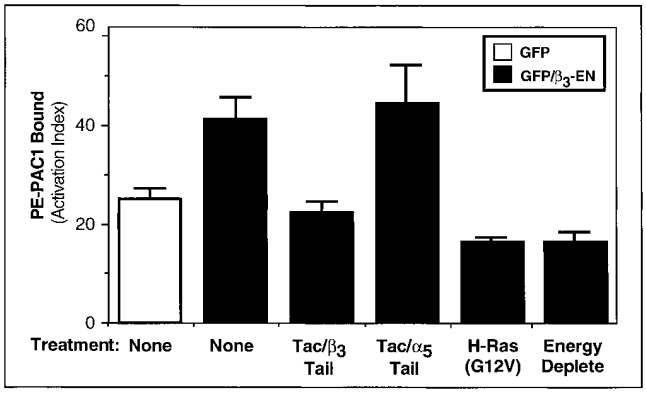

Figure 7. Factors influencing integrin activation by GFP/β_3-endonexin. CHO cells were transfected with $\alpha_{IIb}\beta_3$ αnd either GFP or GFP/β_3-endonexin. As detailed in Materials and Methods, some transfectants were subjected to additional treatments before determination of PAC1 binding. These included (*a*) cotransfection with a Tac/β_3 tail chimera; (*b*) cotransfection with a Tac/α_5 tail chimera; (*c*) cotransfection with a constitutively active form of H-Ras (G12V); or (*d*) energy depletion by preincubation for 30 min with 0.2% sodium azide and 4 mg/ml 2-deoxy-D-glucose. Data are the means ± SEM of three experiments.

Discussion

These studies demonstrate that: (*a*) in CHO cells, expression of β_3-endonexin as a fusion protein with GFP is associated with an increase in the affinity state of integrin $\alpha_{IIb}\beta_3$. This affinity change enables the cells to undergo fibrinogen-dependent aggregation. (*b*) Affinity modulation of $\alpha_{IIb}\beta_3$ by GFP/β_3-endonexin is structurally specific in that other GFP proteins (GFP; GFP/VASP) do not promote this response. Furthermore, GFP/β_3-endonexin does not affect the function of $\alpha_{IIb}\beta_3$ (S752P), a mutant integrin that is defective in binding to β_3-endonexin and in integrin signaling. (*c*) Affinity modulation by β_3-endonexin may be

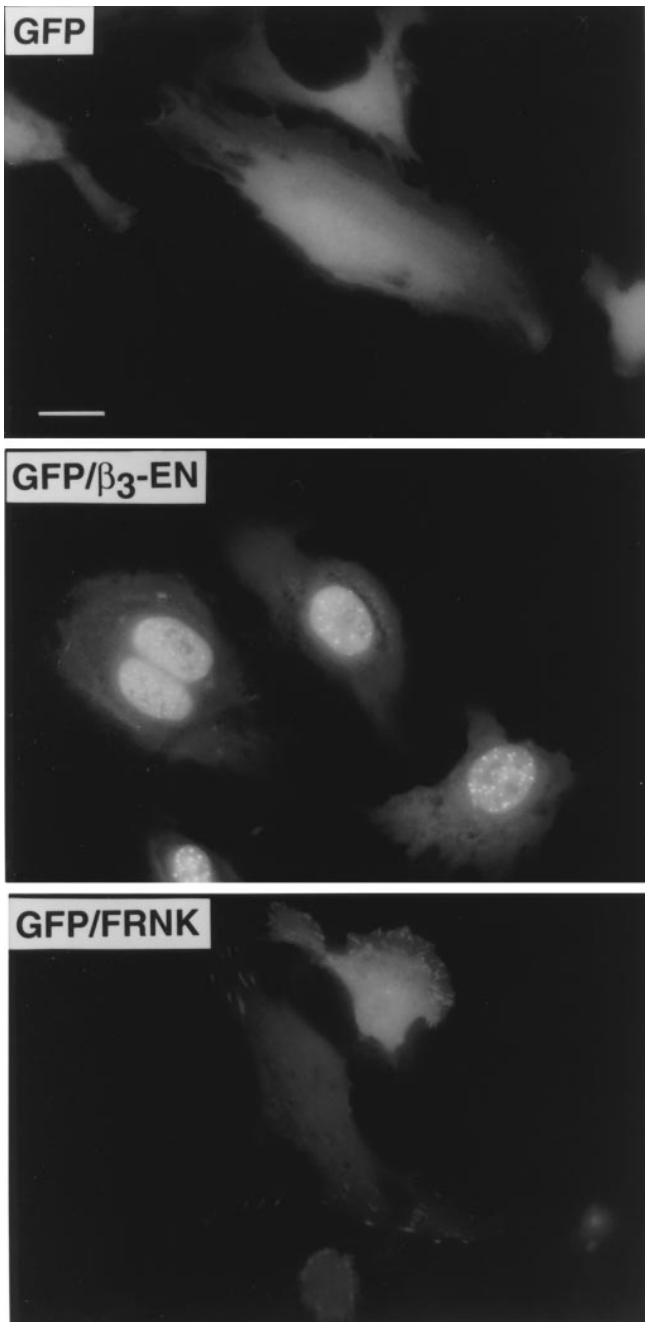

Figure 8. Expression of GFP, GFP/β_3-endonexin, and GFP/VASP in HMEC-1 cells. Cells were allowed to spread for 2 h on fibrinogen-coated coverslips, and then were microinjected with the indicated expression plasmids. 4 h later, green fluorescence was visualized in a fluorescence microscope using an FITC filter set. Uninjected cells were not visible under these conditions. Bar, 10 μm.

the consequence of its direct interaction with $\alpha_{IIb}\beta_3$ since it is prevented by coexpression of a Tac-β_3 cytoplasmic tail chimera. (*d*) The effect of β_3-endonexin on $\alpha_{IIb}\beta_3$ may be subject to metabolic regulation since it is not observed if cellular energy is depleted or if the cells are cotransfected with an activated form of H-Ras. (*e*) GFP/β_3-endonexin is found in both the nuclear and cytoplasmic compartments after CHO cells or HMEC-1 cells have spread on a fibrinogen matrix via a β_3 integrin. Taken together, these results indicate that β_3-endonexin may play a significant role in cell adhesion and signaling through integrin $\alpha_{IIb}\beta_3$.

We interpret the effect of GFP/β_3-endonexin on PAC1 and fibrinogen binding to CHO cells to represent an example of inside-out signaling in which β_3-endonexin increases the affinity of individual $\alpha_{IIb}\beta_3$ heterodimers for specific ligands. An alternative interpretation that β_3-endonexin triggers oligomerization of $\alpha_{IIb}\beta_3$ complexes and therefore increases receptor avidity cannot be excluded, but it seems less likely for several reasons. First, changes within $\alpha_{IIb}\beta_3$ that enable the binding of RGD-containing macromolecular ligands have been detected with both a monovalent Fab fragment of PAC1 as well as with the native, multivalent antibody (1). This indicates that regulated ligand binding to $\alpha_{IIb}\beta_3$ is not absolutely dependent on the valency of the ligand or, presumably, the receptor. Second, the effect of GFP/β_3-endonexin on $\alpha_{IIb}\beta_3$ was detected using either native PAC1 or a monomeric fragment of the antibody. Third, agonist-induced clustering of β_2 integrins in leukocytes and possibly $\alpha_{IIb}\beta_3$ in platelets is facilitated by polymerization of F-actin (12, 14, 71). However, cytochalasin D, an inhibitor of actin polymerization, had no effect on PAC1 binding induced by GFP/β_3-endonexin. While it is not possible to quantitate precisely the relative contributions of affinity and avidity regulation, based on the above considerations, we speculate that β_3-endonexin can regulate reversible fibrinogen binding through affinity modulation. Other factors, including actin polymerization and cytoskeletal reorganization, may enhance cell adhesion by promoting receptor clustering and irreversible ligand binding. Consistent with this idea, cytochalasin D has been reported to inhibit primarily the later, irreversible phase of fibrinogen and PAC1 binding to platelets and CHO cells (14, 53, 54).

The present results were obtained by expressing β_3-endonexin ectopically in CHO cells. Therefore, it is possible that the function of the endogenous protein in platelets or other cells differs quantitatively or qualitatively from that described here. Despite this caveat, a number of observations indicate that affinity modulation may result directly from the interaction of β_3-endonexin with the cytoplasmic tail of the β_3 integrin subunit. A mutational analysis of the β_3 tail has shown that membrane-distal residues near the carboxy terminus of the tail ($N^{756}ITY$) are required for the interaction with β_3-endonexin (13). Mutation or deletion of these same residues also disrupts inside-out integrin signaling in platelets and CHO cells (50; Wang, R., D.R. Ambruso, and P.J. Newman. 1994. *Blood.* 84:244*a*). Moreover, coexpression of a β_3 tail chimera, but not an α_5 tail chimera, prevented affinity modulation by β_3-endonexin, possibly because the former chimera but not the latter could compete with $\alpha_{IIb}\beta_3$ for binding to β_3-endonexin. Finally, when other recombinant GFP proteins such as GFP and GFP/VASP were expressed in CHO cells, they failed to increase $\alpha_{IIb}\beta_3$ affinity.

Additional studies will be required to determine how cellular energy depletion or coexpression of activated H-Ras inhibits affinity modulation by β_3-endonexin. Nonetheless, these results imply that this function of β_3-endonexin is subject to metabolic regulation. In this context, studies with platelets have suggested that serine-thre-

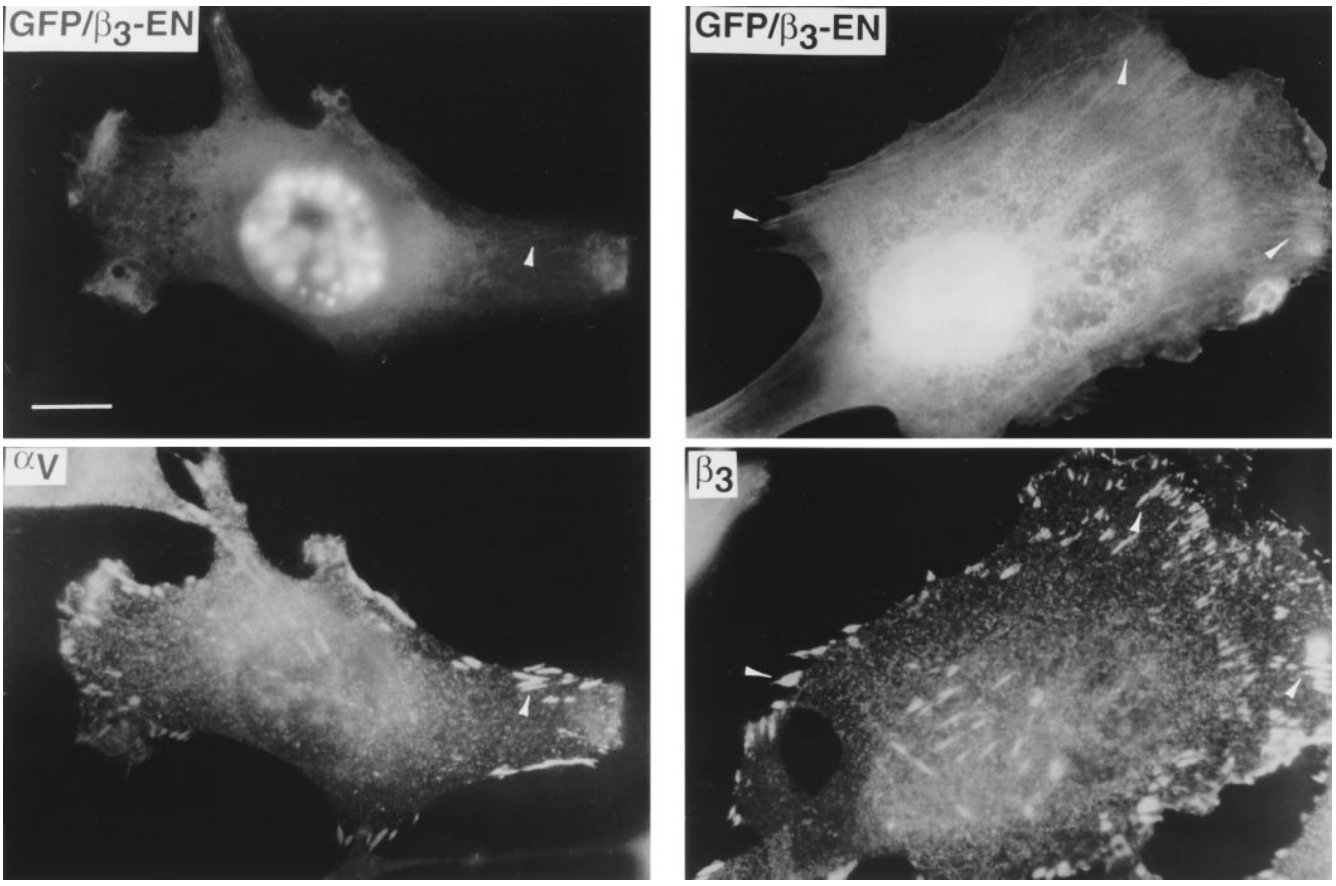

Figure 9. Subcellular localization of GFP/β_3-endonexin and $\alpha_V\beta_3$ in HMEC-1 cells. Cells were allowed to spread for 2 h on fibrinogen and then were microinjected with GFP/β_3-endonexin. 4 h later, the cells were fixed, stained with rhodamine-labeled antibodies to α_V or β_3, and then examined by microscopy for GFP fluorescence (*top*) and rhodamine fluorescence (*bottom*). Two different cells are shown, one in the lefthand panels, the other in the righthand panels. Arrowheads denote the occasional coalignment of GFP/β_3-endonexin and $\alpha_V\beta_3$ in focal adhesions. Bar, 5 μm.

onine kinases (61), tyrosine kinases (23), and PI 3-kinase (38, 74; Kovacsovics, T.J., J.H. Hartwig, L.C. Cantley, and A. Toker. 1995. *Blood.* 86:454*a*) are involved in promoting fibrinogen binding to $\alpha_{IIb}\beta_3$. In contrast, compounds that activate protein kinase A or G inhibit fibrinogen binding (22, 28). Perhaps β_3-endonexin is a direct substrate of specific kinases or phosphatases or is a target of downstream effectors of these enzymes. For example, β_3-endonexin contains several serine and threonine residues in favorable contexts for phosphorylation by protein kinase C and A (63).

Suppression of integrin activation in CHO cells by activated H-Ras involves a Raf-1–initiated MAP kinase pathway and is transcription independent (33). In contrast, an activated variant of R-Ras was recently implicated in the promotion of integrin-mediated cell adhesion (75). Since the reported opposite actions of activated R-Ras and H-Ras affect both β_1 and β_3 integrins, it seems unlikely that the pathways triggered by these GTPases converge directly on β_3-endonexin. Although platelets contain both H-Ras and R-Ras, and platelet stimulation by thrombin activates H-Ras, the functions of these GTPases in this terminally differentiated cell are unknown (64).

Based on the present results, we propose that the interaction of β_3-endonexin with the β_3 cytoplasmic tail triggers a structural change in the integrin to reconfigure the extracellular face of the receptor so that it can engage fibrinogen. While the nature of this propagated change is unknown, one possibility is that there is a reorientation of the β_3 subunit relative to the α_{IIb} subunit. This is plausible given the biophysical evidence for interactions between the cytoplasmic tails of α_{IIb} and β_3 (24) and for agonist-induced structural changes in the extracellular domains of $\alpha_{IIb}\beta_3$ in platelets (65). In a similar manner, relatively subtle changes within preexisting dimers may play a role in ligand-triggered, "outside-in" signaling across other plasma membrane receptors, including the bacterial aspartate receptor (66) and the mammalian EGF receptor (15).

A complete understanding of the proximate events in inside-out signaling will require identification of all relevant integrin-binding proteins and a more refined knowledge of integrin structure. Progress is beginning to be made in both of these areas (11, 42, 55). Several proteins have been described that bind directly to integrin cytoplasmic tails, at least in vitro. These include structural proteins of the cytoskeleton, such as F-actin (specific for the α_2 tail) (36), α-actinin (β tails) (51), talin (β) (29), and filamin (β_2) (60), and potential signaling molecules, such as calreticulin

(α tails) (10), pp125^{FAK} (β) (58), integrin-linked kinase (β) (26), and cytohesin-1 (β_2) (37). Of note, the expression of calreticulin and cytohesin-1 appears to stimulate or stabilize a high affinity state of integrins $\alpha_2\beta_1$ and $\alpha_L\beta_2$, respectively (10, 37). There is no sequence similarity between either of these proteins and β_3-endonexin. Thus, a structurally diverse group of cytoplasmic tail-binding proteins may function to regulate integrins. Some like cytohesin-1 and β_3-endonexin may be restricted in their action because of their binding specificities, while others like calreticulin, which recognizes a conserved motif in all integrin α tails, may be less specific.

While not relevant to platelets, the localization of β_3-endonexin to the cytoplasm and nucleus of HMEC-1 and CHO cells suggests that this protein may have more than one function. The nuclear localization may be explained, in part, by the presence of a consensus nuclear localization signal in β_3-endonexin (K^{62}RKK) (35, 63). Interestingly, several proteins implicated in cell adhesion and adhesive signaling, including ZO-1, β-catenin, zyxin, c-Abl, and HEF1, either exhibit cytoplasmic and nuclear localization or shuttle between the cytoplasm and the nucleus depending on the adhesive state of the cell (Nix, D.A., and M.C. Beckerle. 1995. *Mol. Biol. Cell.* 6:366*a*; 3, 21, 41, 44). The identification of other proteins that can bind to β_3-endonexin should help to explain its pattern of subcellular localization.

Focal adhesions are dynamic structures containing integrins, cytoskeletal elements, and signaling molecules that form on the basal surfaces of many types of cells in culture and in platelets during spreading on fibrinogen (47). These macromolecular assemblies may function to optimize traction during cell motility and to promote information flow from the extracellular matrix to the nucleus (5, 17, 18). The lack of consistent and strong localization of β_3-endonexin to β_3-rich focal adhesions suggests that it may interact most strongly with the β_3 cytoplasmic tail while cells are in suspension or are in the early phases of adhesion. Thus, it is attractive to speculate that β_3-endonexin may participate in integrin activation but may dissociate at later times to permit cytoskeletal interactions with the integrin tails.

These studies provide the first clues about the functions of β_3-endonexin, but they leave several questions unanswered. Does β_3-endonexin influence outside-in signaling events, such as protein tyrosine phosphorylation (8)? Is β_3-endonexin subject to posttranslational modifications in vivo, and does this affect its subcellular localization or function? Does β_3-endonexin modulate the adhesive function of $\alpha_V\beta_3$, which like $\alpha_{IIb}\beta_3$ appears to be subject to rapid regulation in some cell types (67, 73)? Does β_3-endonexin regulate $\alpha_{IIb}\beta_3$ in platelets?

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